While well-studied in DNA, the interaction properties of these compounds with complex RNA structures are less understood. Here we present studies exploring Pt(II) crosslinking in structured RNAs, including a ribozyme and a ribosomal subdomain. In order to isolate and label Pt-bound RNAs, we have developed azide-modified Pt(II) compounds that undergo 'click' chemistry with functionalized fluorophores, biotin, and other reagents. Mapping sites following in vivo exposure demonstrates that Pt(II) preferentially targets purine-rich loops, and fluorescence labeling demonstrates broad binding of these reagents to different cellular RNAs. Taken together, Pt(II) reagents present new opportunities for RNA structure analysis both in vitro and in vivo.

Protein-Nucleic Acid Interactions II

2503-Pos Board B195

Understanding the Role of RNA in Driving the Clustering of HIV Gag Molecules using Coarse-Grained Molecular Dynamics Models

Anand Srivastava, Gregory A. Voth.
Department of Chemistry, Institute for Biophysical Dynamics, James Franck Institute and Computation Institute, University of Chicago, Chicago, IL, USA.

Multi-domain Gag protein is the fundamental building block of the retrovirus particles. In the process of the HIV-1 particle assembly, Gag poly-protein interacts with lipids, with ribonucleic acids (RNAs) and with other Gag proteins. These diverse interactions drive the construction of the infectious particle and the packaging of the viral RNA into the particle. In this work, we have used coarse-grained (CG) molecular dynamics models of plasma membrane, RNA and Gag poly-protein to understand the role of various components in the formation of virus-like particles (VLPs). In particular, we focus on the possible role that the genomic RNA plays as a scaffolding agent in driving the clustering and assembly of Gag proteins on the membrane. We use available CG models of Gag proteins [Ayton & Voth, BJ 2010] and lipids [Srivastava & Voth, JCTC 2012] and develop a new CG model (one-site per nucleotide) for the full genomic RNA using the experimentally available architecture and secondary structure of the entire HIV-1 NL4-3 genome. [Watts et. Al, Nature 2009; Jonikas et al, RNA 2009] Our simulations confirm that the extended Gag molecules are stable only when they are simultaneously associated with both the membrane and the RNA. Using our model, we also show the aggregation of distributed Gag molecules on the membrane surface in presence of RNA strands. Preliminary data from our simulations indicate that existing membrane-bound RNA-Gag seed complexes act as nucleation sites and possibly assist in capturing the diffusing Gag molecule at a distance on the bilayer, slowly increasing the cluster sizes. Large-scale simulation with full genomic RNA is expected to provide further insights into the scaffolding mechanism.

2504-Pos Board B196

Structural Basis for the Mechano-Chemical Coupling and Inter-Subunit Coordination of Ring ATPase

Sara Tafoya[1], Gheorghe Chistol[2], Shixin Liu[1], Paul Jardine[3], Shelley Grimes[3], Carlos Bustamante[1].
[1]University of Californa, Berkeley, San Francisco, CA, USA, [2]Harvard Medical School, Boston, MA, USA, [3]University of Minnesota, Minneapolis, MN, USA.

Members of the Additional Strand Conserved Glutamate (ASCE) superfamily perform a great variety of biological tasks. The gene product 16 (gp16) ring ATPase, one member of the ASCE superfamily, is the active component of the bacteriophage Phi29 packaging motor. Three decades of extensive studies in this system via biochemical and single molecule methods have achieved one of the most comprehensive mechanochemical characterizations of an ASCE ring ATPase to date. The current kinetic understanding of the gp16 ring ATPase provides a solid foundation to build a parallel structural interpretation of its DNA translocation mechanism. It has been shown that the motor translocates DNA using a burst-dwell mechanism and exhibits multiple levels of coordination among the catalytic cycles of individual subunits. Underlying mechanisms, such as inter-subunit communication and proper timing of the cycle by one of the five subunits have been proposed to explain such mechanism. Highly conserved residues such as the arginine finger and the gamma-phosphate sensor as well as important motor-DNA interactions are thought to be responsible for these features; however, the structural mechanism used by this motor is yet to be determined. In this work, we investigate the role of these structural elements in the dynamics of the gp16 ring ATPase by observing DNA translocation in real time using high-resolution optical tweezers and targeted mutagenesis. Our study provides important information regarding the structural design of the gp16 ring ATPase that drives inter-subunit coordination and its coupling to perform DNA translocation. Our results are relevant for other ring NTPases in the ASCE superfamily that share similar structural elements.

2505-Pos Board B197

Structural and Biochemical Studies of the RNA-Associated Sm Protein Superfamily

Cameron Mura.
Department of Chemistry, University of Virginia, Charlottesville, VA, USA.

Sm proteins comprise a broad, evolutionarily conserved family that plays key roles in RNA processing, in organisms ranging from bacteria to human. Eukaryotic Sm proteins form snRNP cores and help organize the RNA splicing machinery, while bacterial Sm proteins (Hfq) interact with small noncoding RNAs to regulate quorum sensing and other sRNA-based pathways; the existence of Sm-like proteins in the Archaea suggests the importance of this ancient family in the early evolution of RNA processing. To decipher the intricate structure ↔ function ↔ evolution relationships in this family, we are pursuing three lines of work that encompass computational analysis and experimental discovery: (i) development of a quantitative 'definition' of the Sm fold, enabling structural analyses and comparison to other small nucleic acid-binding folds, as well as molecular dynamics simulations of Sm proteins; (ii) discovery and identification of small noncoding RNAs bound in vivo by Sm homologs from deep-branching phyla; (iii) crystallographic studies of archaeal Sm proteins and assemblies. Recent results from these directions will be presented.

2506-Pos Board B198

Direct, High-Throughput Measurement of Quantitative RNA Affinity Landscapes

Jason D. Buenrostro, Lauren Chircus, Carlos L. Araya, Curtis Layton, **William J. Greenleaf**.
Stanford University School of Medicine, Stanford, CA, USA.

Evolutionary fitness landscapes are rooted in the biophysical properties of macromolecules under selective pressure. However, the global, comprehensive, and quantitative relationship between sequence permutations and biophysical parameters, such as binding affinity, for simple RNA-protein macromolecular interactions have not been described. Using novel high-throughput methods for generating RNA structures and quantitating protein binding, we measured binding energies for two MS2 viral coat protein variants across millions of target RNA sequences, generating a comprehensive and quantitative corpus of protein-RNA interaction data, including binding energies and off-rates, across RNA variants. These measurements provide extensive information regarding interaction energies with RNA sequence and structure that can be related to salient aspects of crystal structure of the wild-type RNA complexed with the MS2 coat protein. The comprehensive affinity landscape, including all single, double, and triple mutants of the consensus stem loop RNA structure, allowed us to explore RNA hairpin evolvability by examining the effective binding propensities of all quadruple-mutant steps for tens of thousands of evolutionary trajectories. We discover that RNA hairpin evolution is highly constrained, consistent with the wide-spread intramolecular epistasis. Furthermore, we discover that traversable trajectories towards higher affinity are primarily channeled through specific types of mutational changes, providing insight into unique constraints on the evolution of RNA-protein interactions. We anticipate this platform for quantitative RNA biochemistry will provide a powerful tool to the field, providing high-throughput means to probe the relationship between RNA sequence, structure, and function, and thereby matching the combinatorial complexity inherent in RNA sequence variability with equally rich data sets.

2507-Pos Board B199

Engineering Inhibitor Specificity in the Dead-Box Protein Family

Kendall Condon[1], Stephen Floor[1], Jennifer Doudna[1,2].
[1]UC Berkeley, Berkeley, CA, USA, [2]Howard Hughes Medical Institute, Chevy Chase, MD, USA.

The function of both protein-coding and noncoding RNA is dictated by its secondary structure. To maintain this structure, nature has evolved a family of enzymes known as DEAD-box proteins. They are essential to life, most likely due to their diverse roles in RNA splicing, nuclear export and translation, making it difficult to study them using classic genetic methods, like knockouts. Adding to this problem, DEAD-box family members have an extremely conserved active site, hindering the development of chemical inhibitors that will specifically affect one protein and not all those in the family. To circumvent these problems, we have used an approach called the ''gatekeeper'' strategy to engineer inhibitor specificity into DEAD-box proteins. This method involves mutating large residues in the ATP-binding pocket to enlarge it and then engineering an inhibitor that fits this expanded binding site. When the target protein is replaced with its mutant counterpart it becomes susceptible to the inhibitor without cross-reacting with other highly related proteins. Thus, we will be able to observe the effects of inhibition and begin to understand the functional

roles of specific DEAD-box proteins. Here we show the viability of this method for the DEAD-box protein family through both in vitro and in vivo testing of human DDX3X.

2508-Pos Board B200

Characterization of Brr2 Helicase Activity on the U4/U6 snRNAs

Sarah Ledoux, Christine Guthrie.
UCSF, San Francisco, CA, USA.

Brr2 is a member of the DExD/H family of RNA-dependent ATPases that unwinds the extensively base paired U4/U6 snRNAs during pre-mRNA splicing. This reaction is stimulated by the C-terminus of another spliceosomal protein, Prp8. Prp8 has been shown to bind between Brr2's tandem repeat of helicase domains, only the first of which has catalytic activity (1). The lengthy base pairing interaction between U4 and U6 suggests Brr2 must act processively to unwind them. However, there is currently little mechanistic detail describing how Brr2 unwinds the U4/U6 snRNAs.

We are performing a biochemical characterization of the Brr2 helicase activity using a minimal in vitro system. Single turnover ensemble helicase assays demonstrate that the C-terminal fragment of Prp8 increases the overall fraction of U4/U6 snRNAs unwound. However, the Prp8 fragment does not increase the affinity of Brr2 for U4/U6, suggesting Prp8 may increase Brr2's low inherent helicase processivity. Like other helicases, the rate of Brr2 unwinding U4/U6 is highly dependent upon the monovalent cation concentration, decreasing exponentially as the concentration of salt increases. Mutations within the putative ''unwinding ratchet'' of Brr2 result in a lower extent of total U4/U6 snRNAs unwound. We predict that these mutations decrease the processivity of Brr2 and result in more aborted attempts at unwinding. Using the conditions established in our ensemble assays, we are now performing single molecule Forster Resonance Energy Transfer (smFRET) assays to further interrogate the mechanism of Brr2 helicase activity and its stimulation by Prp8.

(1)Santos, K.F. et al., Proc Natl Acad Sci U S A 109 (43), 17418-17423.

2509-Pos Board B201

Development of an smFRET Assay to Monitor Spliceosome Activation in Budding Yeast

Megan Mayerle, John Abelson, Christine Guthrie.
Biochemistry and Biophysics, University of California San Francisco, San Francisco, CA, USA.

The addition of the U4/U6-U5 tri-snRNP to the spliceosome instigates many changes to create a catalytically active spliceosome. Specifically the U6 internal stem-loop (ISL), structurally reminiscent of catalytic domain V of self-splicing group II introns, must form. U6 arrives at pre-mRNA stably base-paired with U4 in the tri-snRNP. The U4/U6 interaction sequesters the U6 ISL, blocking premature activity. Unwinding, regulated by proteins Brr2 and Prp8, allows for mutually exclusive U2/U6 interactions required for catalysis to form. This ensures assembly is highly coordinated while allowing for the careful regulation required for fidelity. Because most splicing studies use crude extracts, wherein multiple reversible steps in spliceosome assembly proceed asynchronously, fundamental details of the kinetics and conformational rearrangements undergone by U6 during activation are unknown.

Single-molecule FRET provides a method to monitor dynamics and conformation without isolating or synchronizing reaction intermediates. I am currently developing an smFRET assay to monitor U6 ISL formation as a readout for U4/U6 unwinding in spliceosome activation. I have created a dual-fluorophore labeled U6 RNA construct that enables me to discern whether the U6 ISL is formed or sequestered by U4. This construct is incorporated into U6 snRNPs and is functional in in vitro splicing assays. To facilitate imaging, I have optimized a protocol to isolate tri-snRNPs. Once optimization of this assay is complete, I will determine the kinetics and molecular mechanism of how Brr2 unwinds U4/U6.

2510-Pos Board B202

Reconstitution and Single Molecule Characterization of Yeast Spliceosomal E Complex

Sarah Hansen[1], Margaret Rodgers[1], Ross Laurent[1], Yuliang Sun[1], Mark Scalf[2], Lloyd Smith[2], Aaron Hoskins[1].
[1]Biochemistry, University of Wisconsin Madison, Madison, WI, USA, [2]Chemistry, University of Wisconsin Madison, Madison, WI, USA.

The formation of yeast commitment complex (CC2 or ''E complex'') is one of the earliest stages in spliceosome assembly on pre-mRNA, and is the only assembly step that forms independent of the activity of an ATPase. Previous studies have shown that E complex components, the nuclear cap binding complex (CBC) and the branchpoint binding Msl5•Mud2 heterodimer, are required to stabilize U1 small nuclear ribonucleoprotein (snRNP) complex to the 5′SS of pre-mRNA. However the kinetics and nature of the interactions between these components have not been established, in part because E complex has never been reconstituted separate from other spliceosomal proteins. Here we present work towards the characterization of E complex interactions by Colocalization Single Molecule Spectroscopy (CoSMoS) experiments with reconstituted fluorescently-labeled CBC, Msl5•Mud2, and U1 snRNP. Intact and functional U1 snRNP was isolated from all other spliceosomal proteins by a novel purification method and fluorescently labeled with a trifunctional SNAP-ligand derivative for single molecule study. The stoichiometry of the seventeen U1 snRNP proteins and the efficiency of the fluorescent labeling reaction is being determined by AQUA mass spectrometry. A combination of splicing assays, primer extension, and CoSMoS experiments with fluorescently labeled pre-mRNA constructs are used to show the functionality of purified U1 snRNP. Our results show that the multi-protein components of E complex can be reconstituted and fluorescently tagged for CoSMoS and other single molecule experiments which elucidate the kinetics of early spliceosome assembly.

2511-Pos Board B203

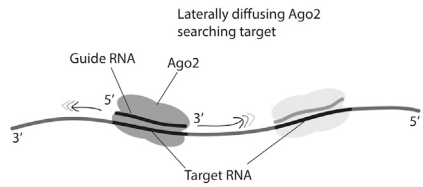
Weak Interactions between Risc and mRNA Promote Optimal Targeting in RNA Interference

Stanley Dinesh Chandradoss[1], Ian MacRae[2], Chirlmin Joo[1].
[1]TU Delft, Delft, Netherlands, [2]The Scripps Research Institute, San Diego, CA, USA.

RNA interference (RNAi) is an essential regulatory process in embryonic development, tumor genesis and other important functions in eukaryotic cells. RNAi occurs when the Argonaute (Ago) protein brings a small non-coding RNA, such as siRNA (small interfering RNA), to target mRNA. Despite a decade of research, it remains an open question how Ago mediates specific targeting inside a crowded cell while it does not utilize any external energy such as ATP.

Here we used a single-molecule FRET technique to visualize the target search process of RISC (RNA-induced silencing complex), which consists of human Ago2 and siRNA. We observed that RISC dynamically binds to and dissociates from mRNA. This dynamic interaction occurs even when there is substantial basepairing between siRNA and mRNA. RISC that dissociates from its first target is able to diffuse laterally along the RNA strand and pair with another target nearby.

This dynamic search process will lead to an optimal match among many potential pairs. It hints that RISC may have evolved to compromise the search time for the target recognition fidelity. The outcome of our research will be useful in eliminating off-targeting in RNA interference.



2512-Pos Board B204

A Conserved Mechanism of RNA Substrate Recognition and Cleavage by Fungal Dicers

Mary Anne Kidwell, Jessica M. Chan, Jennifer A. Doudna.
MCB, UC Berkeley, Berkeley, CA, USA.

Dicer is a central enzyme for processing small RNAs in RNA interference. It is able to cleave both hairpin and long double-stranded RNA precursors into microRNAs and small interfering RNAs respectively. While humans have one Dicer that is able to process both substrates, many organisms have multiple copies. Understanding how organisms with two Dicers chose specific RNAs for cleavage could give insight into how the human protein functions.

We have turned to the thermophilic fungus *Sporotrichum thermophile* as a model system to purify stable Dicers for biochemical dissection. While the function of these two Dicers was thought to be partially redundant, our results show that they cleave different RNA substrates, similar to what is observed in the *Drosophila melanogaster* system. *S. thermophile* Dicer-1 dices hairpin precursor microRNAs faster than long double-stranded RNAs while *S. thermophile* Dicer-2 can only cleave perfect RNA duplexes accurately with the requirement for ATP. Removal of the helicase domain from Dicer-2 relieves the requirement of ATP for cleavage and allows the enzyme to process hairpin substrates. In addition, only Dicer-2 shows robust ATP hydrolysis in the presence of dsRNA and partial stimulation in the presence of single-stranded RNA or an RNA/DNA hybrid. The conserved function of these two Dicers may indicate a common mechanism used by eukaryotes to process small RNAs.